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Novel bifunctional probe for radioisotope-free photoaffinity labeling: compact structure comprised of photospecific ligand ligation and detectable tag anchoring units †

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A novel method for radioisotope-free photoaffinity labeling was developed, in which a bifunctional ligand is connected to a target protein by activation of a photoreactive group, such as an aromatic azido or 3-trifluoromethyl-3*H*-diazirin-3-yl group, and identification of the ligated product is achieved by anchoring of a detectable tag through the Staudinger–Bertozzi reaction with an alkyl azido moiety that survives photolysis. The chemical ground of this method was confirmed using model compounds with the bifunctional group under photoirradiation in the presence of trapping agents for reactive intermediates. The utility of the method has been demonstrated by specific labeling of the catalytic portion of human HMG-CoA reductase.

The discovery of new proteins with specific functions is a central theme in post-genomic science. Photoaffinity labeling (PAL)[1,2] has become a powerful method in this area as confirmed by the identification of target proteins of biologically active molecules and elucidation of their binding sites. Therefore, PAL offers good potential for discovery and development of drugs.[3] PAL studies focus on how to design an efficient photoaffinity probe satisfying the following three requirements: (1) strong and specific binding with a target protein, (2) high photospecificity to allow the efficient formation of a covalent bond between the ligand and its target protein, and (3) high sensitivity of detection. Photoreactive functional groups, such as azido, diazo, diazirinyl, and benzophenone, and radioisotopes (RIs), such as ^{3}H, ^{14}C, ^{32}P, ^{35}S, and ^{125}I, are frequently used for the second and third purposes. With the increase in importance of PAL, novel strategies that do not require the use of RIs are urgently needed. Photoaffinity biotinylation is a representative non-RI-based labeling protocol.[4] This method utilizes strong avidin–biotin complex formation to allow harmless chemiluminescent detection and direct affinity purification of a labeled protein for sequencing. However, the introduction of a highly polar and sterically congested biotin-anchored tag to an affinity compound often results in marked impairment of intrinsic biological activity in the crucial probe design step. This paper presents a novel protocol for RI-free PAL based on a compact bifunctional probe unit capable of differentiating between ligand ligation and labeled protein detection.

The concept of our method based on the design of a bifunctional photoaffinity probe equipped with a highly photosensitive group, such as an aromatic azido or 3-trifluoromethyl-3*H*-diazirin-3-yl group, and a relatively photostable alkyl azido group is illustrated in Fig. 1.[5] We anticipated that an alkyl azido group could be left intact under conditions for photoactivation of an aryl azido or 3-trifluoromethyl-3*H*-diazirin-3-yl group (Step 1), and could therefore be used as a tag for consecutive detection of the covalently cross-linked protein.[6,7] Thus, we envisaged that the remaining alkyl azido group could be labeled chemoselectively by Staudinger–Bertozzi ligation[8] using a fluorescent marker-[9] or biotin-anchored[8] triarylphosphine derivative (Step 2).[10] We also considered that the introduction of a small azidomethyl group would be preferable to minimize steric effects and changes in polarity that may adversely influence the bioactivity. Furthermore, the additional azido and azidomethyl groups would be placed in *meta* positions to avoid unnecessary interactions with the three neighboring functional groups on the aromatic ring.[11]

First, we conducted model experiments using azido- and trifluoromethyldiazirinyl-functionalized compounds, **1** and **5**, to verify the tolerance of the alkyl azido group under the conditions of photoactivation adaptable to the aryl azido and diazirinyl photo-functions (Scheme 1). Thus, a C_2-symmetrical triazido-functionalized compound **1** (20 mM in cyclohexane-d_{12})[12] and a 300-fold excess of diethylamine were put in a quartz NMR tube and exposed to UV with a wavelength of 254 nm (Asahi Spectra, LAX-101, 100 W) at 22 °C (Scheme 1a). The course of the reaction was monitored by ^{1}H-NMR. Disappearance of **1** was accompanied by a significant set of new spectra. Consequently, irradiation for 8 h afforded **4** in 56% yield, which is equivalent to 64% yield based on the consumption of **1**. The product was isolated and characterized as the diethylamino-substituted 3*H*-azepine **4**, obtained by nucleophilic addition of diethylamine to didehydroazepine intermediate **3** produced by ring expansion of photo-generated singlet nitrene species **2**.[2b,5,13] The above observations indicated that most of the alkyl azido groups remained intact under the conditions for photoactivation of the aromatic azido group.[14] Similarly, the diazirinyl-type compound **5** (3.0 mg/0.75 mL CD_3OD) underwent photoreaction in a quartz NMR tube by continuous irradiation with UV at 365 nm (UVP, UVL-56, 6 W) for 10 min and 302 nm (UVP, UVM-57, 6 W) for 8 min at 22 °C (Scheme 1b).[15] On ^{19}F-NMR, the peak corresponding to **5** (δ 10.8 ppm)

† Electronic supplementary information (ESI) available: Photodecomposition study of phenyl and benzyl azides. Experimental details for photoreactions of **1** and **5**. Synthetic procedures and characterization of new compounds. See http://www.rsc.org/suppdata/ob/b3/b316221d/.

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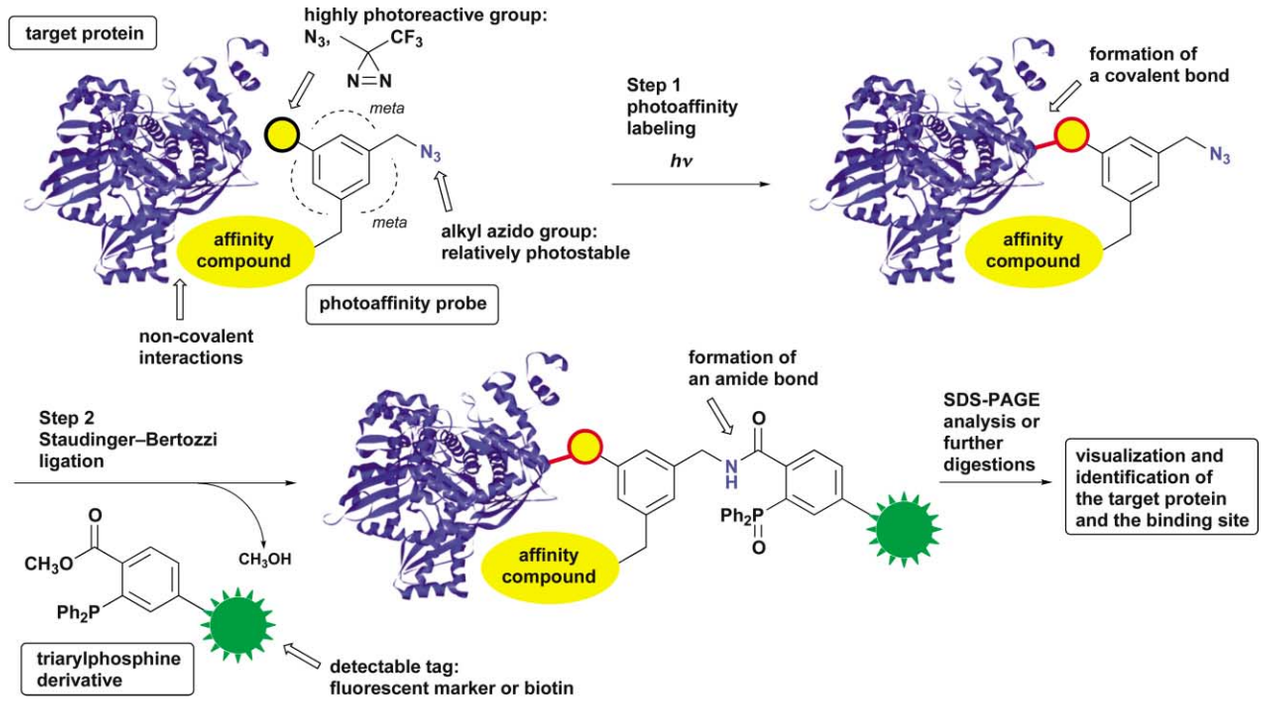
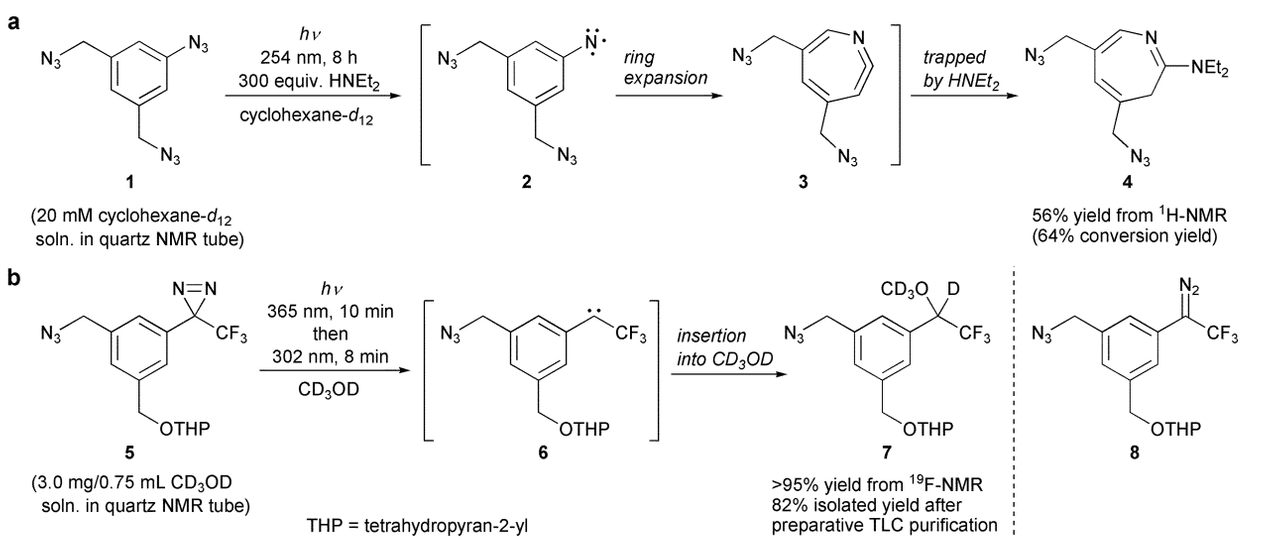


Fig. 1 Concept for radioisotope-free photoaffinity labeling using a specific ligand with bifunctional groups. The photoreaction realizes formation of a covalent bond between the ligand and target protein (step 1) and consecutive Staudinger–Bertozzi ligation of the detectable tag allows visualization of the captured protein (step 2).



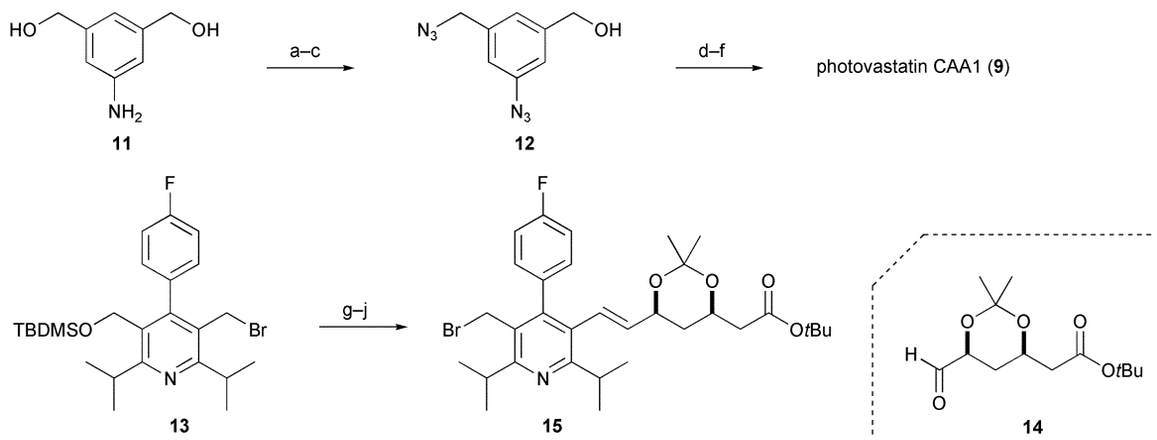
Scheme 1 Photoreactions in model compounds to confirm functional discrimination.

disappeared completely and a new peak corresponding to the adduct **7** appeared at δ −0.4 ppm. Adduct **7** was produced by insertion of the photo-generated carbene species **6** into CD_3OD and was almost the sole product (>95% according to integration).[4f,16] Adduct **7** was isolated by preparative TLC in 82% yield. Thus, we showed that the aryl azido and diazirinyl units are preferably photoactivated to the alkyl azido group under the above conditions, and therefore, the latter alkyl azido functional group could be utilized as trigger component for Staudinger–Bertozzi ligation in both cases.

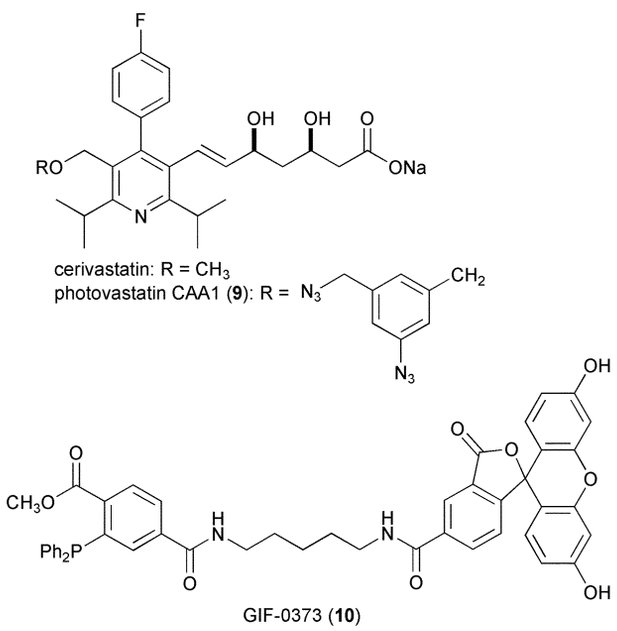
Encouraged by the positive verification in the model reaction, we set up an actual PAL protocol to confirm our concept. We chose to photolabel the catalytic portion of human 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase using a diazido-functionalized cerivastatin derivative, referred to as photovastatin CAA1 (**9**), and then fluorescein-anchored triarylphosphine derivative, GIF-0373 (**10**).

HMG-CoA reductase (HMGR) is the rate-limiting enzyme in the cholesterol biosynthetic pathway, which converts HMG-CoA to mevalonate.[17] Cerivastatin[18] and other therapeutically useful statins inhibit HMGR activity by binding to its active site[19] to decrease cholesterol biosynthesis, thus reducing the risk of coronary heart disease.[20] In contrast to their clinical efficacy, statins occasionally trigger harmful side effects, such as rhabdomyolysis.[21] A high incidence of this fatal myopathy was induced by treatment with cerivastatin, prompting removal of this statin from the global market.[22] The molecular mechanism underlying statin-induced myopathy is still not clear.[20,23] Promising pleiotropic effects of statins on other diseases, such as osteoporosis, dementia, and multiple sclerosis, have attracted a great deal of attention in recent years,[24] leading to the demand for elucidation of the mechanism of their severe adverse effects in association with the expansion of the application of these molecules and new drug discovery. Our photoaffinity probe **9** was also designed with the aim of identifying the key molecule that triggers the myopathy.

In designing **9**, we replaced the methoxy moiety of cerivastatin, which is the most distinctive structural unit among the



Scheme 2 Synthesis of photovastatin CAA1 (**9**). (a) $NaNO_2$, $AcOH/H_2O$ (9 : 1), 0 °C, 3 min, then NaN_3, 0 °C, 15 min, 88%; (b) CBr_4, PPh_3, DMF, 0 °C, 2 h, 53%; (c) NaN_3, DMF, 0 °C to room temperature, 6.5 h, 96%; (d) NaH, DMF, 0 °C, 15 min, then **15**, 0 °C, 3 h, 59%; (e) 10% aq. HCl/THF (1:4), room temperature, 14 h, 91%; (f) aq. NaOH, THF, room temperature, 11.5 h, 93%; (g) $Ph_2P(OEt)$, 150 °C, 3 h, 99%; (h) *n*BuLi, THF, 0 °C, 20 min, then **14**, 0 °C, 4 h, 66%; (i) nBu_4NF, THF, room temperature, 5 h, 98%; (j) CBr_4, PPh_3, CH_2Cl_2, room temperature, 1 h, 71%. TBDMS = *tert*-butyldimethylsilyl.



statins, by the photoactivatable benzyloxy group. We chose the sterically less congested diazido derivative as a representative to minimize the change of the biological function of the original compound. As shown in Scheme 2, the synthesis of **9** was accomplished by coupling the diazido-functionalized benzyl alcohol **12** with bromide **15**, derived from known components **11**[25] and **13**,[26] respectively, under conventional conditions, followed by successive deprotections of acetal and ester portions. The inhibitory effect of synthesized **9** on the catalytic portion (residues 426–888) of recombinant human HMGR ($HMGR^{426–888}$) was assayed.[27] Under our assay conditions,[28] the IC_{50} values for **9** and cerivastatin were determined to be 0.95 nM and 0.93 nM, respectively. Thus, we judged that **9** could be used as a photoaffinity probe.

Using the promising photoaffinity probe **9**, we next carried out the actual PAL experiment according to the protocol shown in Fig. 1 as follows: (1) incubation of **9** in various concentrations (final concentration, 2, 5, 20, 50 nM) with $HMGR^{426–888}$ for 5–10 min at room temperature in the absence or presence of cerivastatin (final concentration, 5 μM),[29] (2) photoirradiation of the mixtures using a portable UV-lamp (UVP, UVG-54, 254 nm, 6 W, 60 s × 2, from a distance of 1 cm, room temperature), (3) Staudinger–Bertozzi ligation by adding an excess amount of **10** (100 μM) to the mixtures and incubation for 4 h at room temperature, (4) denaturation of $HMGR^{426–888}$ in the reaction mixture for 1–2 min at 95 °C in 0.1 M Tris-Cl (pH 6.8) containing 1% sodium dodecylsulfate (SDS) and 2.5% β-mercaptoethanol, and (5) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the mixtures using 12.5% polyacrylamide gels in the Laemmli buffer system.[30] As shown in Fig. 2, the gels were directly visualized by laser-scanning in a fluorescent imaging analyzer.[31] As can be seen from lanes 1, 3, 5, and 7, $HMGR^{426–888}$, with a calculated molecular mass of 51 kDa, was successfully photolabeled by **9** in a dose-dependent manner. It is worth noting that the labeled signal was detectable even when **9** was employed at a concentration of 2 nM (lane 1), which is close to its IC_{50} value. All of the fluorescent signals were decreased by coincubation with

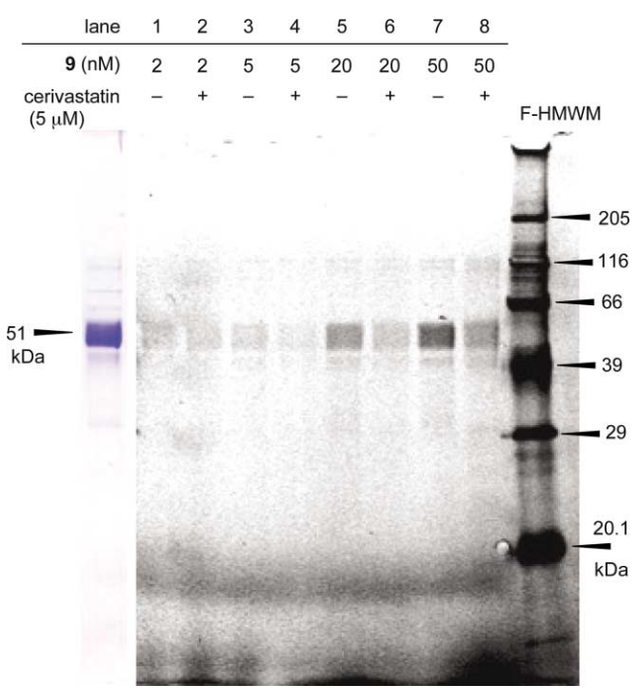


Fig. 2 SDS-PAGE analysis of $HMGR^{426–888}$ (51 kDa) photolabeled in various concentrations of **9** in the absence (−) or presence (+) of cerivastatin (5 μM) followed by chemoselective ligation with **10** (100 μM). The fluorescent signal was visualized by laser-scanning of the electrophoresed gel with a fluorescent imaging analyzer (FluorImager SI, Molecular Dynamics, 488 nm excitation, 530 ± 15 nm detection filter). For comparison, Coomassie brilliant blue (CBB)-stained $HMGR^{426–888}$, which was transferred onto a polyvinylidene difluoride (PVDF) membrane after SDS-PAGE, is shown in the leftmost lane. The rightmost lane shows the fluorescent high molecular weight marker (F-HMWM, Sigma).

excess amounts of cerivastatin, indicating that the cross-linking proceeded specifically at the active binding site (lanes 2, 4, 6, 8).[32]

Next, we identified the site of cross-linkage of **9** on HMGR by digesting the photolabeled protein.[33] Low molecular weight peptide fragments (<10 kDa) were analyzed by Tris-tricine SDS-PAGE.[34] Among the proteolytic enzymes examined, lysylendopeptidase (LEP), which specifically cleaves the peptide bond at the C-terminal of lysine residues, gave the best result. As shown in Fig. 3a, after transferring proteins from the gel onto the polyvinylidene difluoride (PVDF) membrane, the fluorescent signals were visualized using a UV transilluminator. As can be seen from lanes 1 and 2 in Fig. 3a, two sharp fluorescent bands, referred to as A and B, were observed.[35] After staining with Coomassie brilliant blue (CBB) (Fig. 3b), the selected peptide fragments, bands A–E in Fig. 3, were cut out and subjected directly to protein sequence analysis. The first five N-terminal amino acid residues identifiable from bands A and C were D-N-P-G-E- and L-S-E-P-S- corresponding to residues 829–833 and 503–507, respectively, of human HMGR.[36] On the other hand, band B contained an approximately 1:4 mixture of fragments starting with D-N-P-G-E- and L-S-E-P-S-.[37] As band C was derived from a mixture without photoreaction with **9**, both fluorescent bands were mostly attributed to the same fragment starting with D-N-P-G-E-. The different lengths of these bands could be explained by the alternative digestion patterns by LEP. The fluorescence intensity of these two bands differed depending on whether the photoreaction was performed in the presence or absence of NADPH (lanes 1 and 2 in Fig. 3a, respectively), in which the signal for the fragment with lower molecular weight, band B in lane 1, relatively decreased. Because it is elucidated

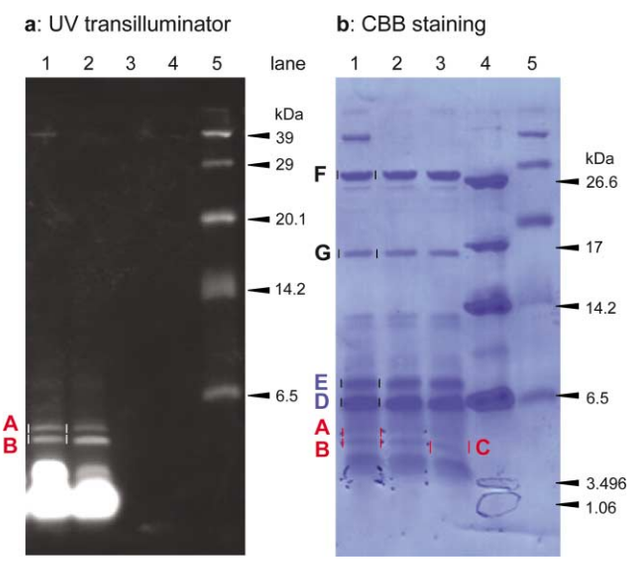


Fig. 3 Tris-tricine SDS-PAGE analysis of digested HMGR$^{426–888}$ peptide fragments obtained by photolabeling with **9** (5 μM) followed by ligation with **10** (100 μM) and then treatment with LEP (Wako). (a) The photograph was taken with a monochrome CCD camera (ATTO, Printgraph AE-6914) after transferring proteins from the gel onto a PVDF membrane, and visualization on a UV transilluminator (UVP, TLW-20, 365 nm, 8 W × 4). (b) The same membrane after CBB staining. Lanes 1 and 2 represent peptide fragments from the photoreaction in the presence or absence of 250 μM NADPH, respectively. Lane 3 shows the control experiment for proteolysis of HMGR$^{426–888}$ without photoreaction with **9**. Lanes 4 and 5 indicate ultra-low range molecular weight marker (Sigma) and fluorescent low molecular weight marker (Sigma), respectively. The dots in lanes 1 and 2 and the circles in lane 4 in the CBB-stained membrane are pencil marks made to pinpoint the positions of fluorescent bands and the molecular weight marker, respectively. Fragments A–E were cut out of the CBB-stained gel and subjected to direct protein sequence analysis (Shimadzu, Automated Protein/Peptide Sequencer PPSQ-23A). Bands F and G are from LEP.

from kinetic and structural studies that NADPH does not share its binding site on HMGR with statins,[38] this co-enzyme would be invoking some conformational change on stain-photolabeled HMGR complex to confer some resistance to proteolysis, although the precise effect of NADPH is not yet clear. These results demonstrated the specific cross-linking of **9** at the C-terminal domain of HMGR, as expected based on the X-ray co-crystallographic structures of HMGR and cerivastatin.[19,39]

In the present study, we developed a novel chemical methodology for RI-free PAL. This protocol may also be utilized not only for other types of photoreactive functions, such as nitro- or perfluoro-substituted aryl azides and benzophenone derivatives to modulate the efficiency of the ligand–protein ligation, but may also allow the introduction of an alkyl azido group anywhere in a ligand structure to increase structural diversity for suitable matching of binding and activity.[40] Further studies on PAL using another diazirinyl unit as a photoreactive function and the exploration of target proteins involved in statin-associated myopathy based on the versatile PAL method will be reported in due course.

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27 A 1.4-kb DNA encoding human HMGR$^{426–888}$ was obtained by reverse transcription-polymerase chain reaction (RT-PCR) from total RNA of HepG2 cells and then subcloned into the pQE30 expression plasmid (QIAGEN) to express an N-terminal hexahistidine-tagged protein. The recombinant protein was expressed in *E. coli* JM109 cells at 37 °C followed by purification using a TALON affinity matrix (Clontech). The nearly homogeneous protein was stored in a buffer containing 50 mM sodium phosphate (pH 7.0), 0.3 M NaCl, 10 mM dithiothreitol (DTT), and 10% glycerol at −20 °C. The stocked protein solution was used directly for enzyme assay and photolabeling studies in its histidine-tagged form without further modification.

28 The enzyme assays to determine the values of median inhibitory concentrations (IC_{50}) of **9** and cerivastatin for HMGR were performed by monitoring the (*R*,*S*)-HMG-CoA-dependent oxidation of NADPH at 340 nm in an absorbance microplate reader (Tecan, Sunrise) at 30 °C for 18 min. Standard assay mixtures placed on the 96-well plate contained, in a final volume of 100 μL, 2.5 μL DMSO solution of **9** or cerivastatin in various concentrations, 24 ng HMGR$^{426–888}$, 60 μM (*R*,*S*)-HMG-CoA (Sigma), 250 μM NADPH (Oriental Yeast), 75 mM NaCl, 1 mM EDTA, 10 mM DTT, and 0.1 M sodium phosphate buffer (pH 7.0). The IC_{50} values were obtained as the averages of at least three runs.

29 PAL experiments were performed under conditions similar to those used in the enzyme assay. The reaction mixtures contained, in a final volume of 20 μL, 0.2 μL DMSO solution of **9** in various concentrations with or without 0.2 μL DMSO solution of cerivastatin (5 μM), 1 μg HMGR$^{426–888}$, 60 μM (*R*,*S*)-HMG-CoA, 250 μM NADPH, 0.2 M NaCl, and 20 mM PIPES/Tris buffer (pH 7.0).

30 U. K. Laemmli, *Nature*, 1970, **227**, 680–685.

31 Fluorescent signals were similarly observed after transferring proteins from the gel onto PVDF membranes. PAL using >50 nM of **9** allowed detection of the fluorescent signal on a UV transilluminator.

32 Not significant but slight fluorescent signal was observed even when there was no UVirradiation. See ESI for the detail. † Staudinger–Bertozzi ligation of photolabeled protein with a biotin-anchored triarylphosphine derivative and its chemiluminescent detection by streptavidin–horseradish peroxidase was also effective.

33 Increasing the amounts of **9** and enzyme used made the analysis easier. Thus, the reaction mixtures for LEP treatment contained, in a final volume of 40 μL, 0.4 μL DMSO solution of **9** (5 μM), 0.4 μL DMSO solution of cerivastatin (500 μM), 10 μg HMGR$^{426–888}$, 60 μM (*R*,*S*)-HMG-CoA, 250 μM NADPH, 0.2 M NaCl, and 20 mM PIPES/Tris buffer (pH 7.0). The mixture was photoirradiated for 60 s × 2, followed by addition of **10** (100 μM) and incubation for 30–60 min. This was then followed by the successive addition of SDS (a final volume, 0.1%) and 5 μg of LEP. The mixture was incubated at 37 °C for 24 h and then subjected to Tris-tricine SDS-PAGE analysis.

34 H. Schägger and G. von Jagow, *Anal. Biochem.*, 1987, **166**, 368–379.

35 The fluorescent intensities of these bands were significantly decreased by co-incubation with a hundredfold greater amount of cerivastatin. The lower molecular weight fragments (<4 kDa) were superimposed with the broad low molecular weight band including unreacted **10**.

36 For the full amino acid sequence of human HMGR consisting of 888 amino acids, see, K. L. Luskey and B. Stevens, *J. Biol. Chem.*, 1985, **260**, 10271–10277.

37 Non-florescent but CBB-stained bands D and E were shown to start with E-F-Q-V-P- and N-L-V-G-S-, which correspond to residues 550–554 and 736–740, respectively.

38 (*a*) A. Endo, M. Kuroda and K. Tanzawa, *FEBS Lett.*, 1976, **72**, 323–326; (*b*) E. S. Istvan, M. Palnitkar, S. K. Buchanan and J. Deisenhofer, *EMBO J.*, 2000, **19**, 819–830; (*c*) E. S. Istvan and J. Deisenhofer, *Biochim. Biophys. Acta*, 2000, **1529**, 9–18.

39 Protein Data Bank, accession number 1HWJ.

40 Bioconjugation by azide–alkyne [3+2] cycloaddition instead of Staudinger–Bertozzi ligation would be also applicable, see, (*a*) A. J. Link and D. A. Tirrell, *J. Am. Chem. Soc.*, 2003, **125**, 11164–11165; (*b*) A. Dieters, T. A. Cropp, M. Mukherji, J. W. Chin, C. Anderson and P. G. Schultz, *J. Am. Chem. Soc.*, 2003, **125**, 11782–11783; (*c*) R. Breinbauer and M. Köhn, *ChemBioChem.*, 2003, **4**, 1147–1149 and references therein.